VIROLOGY 213, 405–412 (1995)

# Phosphorylation of Ser$^{232}$ Directly Regulates the Transcriptional Activity of the P Protein



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*Received July 7, 1995; accepted August 24, 1995*

The phosphoprotein P of human respiratory syncytial virus (RSV) was expressed in eukaryotic cells in phosphorylated form. Site-directed mutagenesis of the recombinant protein established Ser$^{232}$ as the major site of phosphorylation *in vivo.* Phosphorylation of bacterially made P protein *in vitro* by purified casein kinase II (CKII) resulted in the phosphorylation of Ser$^{237}$, whereas mainly Ser$^{232}$ was phosphorylated by a crude cell extract. The P kinase activity in the cell extract exhibited properties characteristic of CKII. While the Ser$^{232,237}$ to Ala double mutant was nearly completely defective for phosphorylation and transcription, phosphorylation at Ser$^{232}$, through the use of appropriate P mutant or kinase, activated P protein. Phosphorylation of Ser$^{237}$ restored activity only to the extent it facilitated phosphorylation of Ser$^{232}$. Phosphate groups of P protein in RSV-infected cells were highly stable; inhibitors of protein serine phosphatases had no effect on the intracellular turnover of the phosphates. Highly purified viral polymerase L was transcriptionally active but devoid of P protein kinase activity. Thus, CKII-mediated phosphorylation of Ser$^{232}$ appears to be the primary regulator of P protein activity while phosphorylation of Ser$^{237}$ may be involved in a modulatory role under certain conditions. 

## INTRODUCTION

Human respiratory syncytial virus (RSV) is the leading cause of respiratory illness and death in young infants worldwide (Heilman, 1990; Hall, 1994). It is the prototype member of the *pneumovirus* genus in the family *Paramyxoviridae* and contains a nonsegmented negative-strand RNA genome about 15 kb long. The 10 characterized genes are arranged in the linear single-stranded genome in the order: $^{3'}$1C-1B-N-P-F-M-G-SH-22K-L$^{5'}$ (reviewed by Collins, 1991). While the mechanism of viral gene expression is not known in detail, we have recently shown that an active viral transcription complex could be reconstituted *in vitro* using the N-RNA template (genomic RNA complexed with nucleocapsid protein N), the viral L protein, and phosphorylated viral P protein (Mazumder and Barik, 1994). In addition, functional transcription required cellular protein(s), at least one of which was actin (Barik, 1992; Huang *et al.,* 1993; Mazumder and Barik, 1994). The *in vitro* system was then exploited to address the role of phosphorylation of the P protein. By substituting the viral P protein with bacterially expressed, phosphate-free P protein in the reconstitution reaction, we demonstrated an essential role of phosphorylation by cellular casein kinase II (CKII) in the transcriptional activity of the P protein (Mazumder and Barik, 1994). Deletion studies of the recombinant protein (Mazumder *et al.,* 1994) as well as proteolytic analysis of the viral P protein (Villanueva *et al.,* 1994) located the phosphate groups near the C-terminus of the protein. Moreover, use of recombinant P proteins mutated at specific Ser residues resulted in the identification of Ser$^{237}$ as a major site of phosphorylation by purified CKII *in vitro* (Mazumder *et al.,* 1994). In the present communication, we have continued these studies further and investigated the nature of phosphorylation of P protein *in vivo,* its possible phosphorylation by viral L protein, and the transcriptional activity of various phosphorylated species of P protein.

## MATERIALS AND METHODS

### Cloning and expression of P gene and its mutants

RSV (long) P gene and its mutants were PCR-amplified from the corresponding bacterial (pET-3a) clones constructed previously (Mazumder *et al.,* 1994), and subcloned between *Bam*HI and *Xba*I sites of pcDNA3 (Invitrogen, San Diego, CA). A 70% confluent monolayer of HEp-2 cells in a 33-mm diameter petri dish was transfected with 5 $\mu$g of the pcDNA3-P DNA using Lipofectin (Gibco-BRL, Gaithersburg, MD) according to manufacturer's protocol. Metabolic labeling of the transfected cells with [$^{32}$P]orthophosphate and immunoprecipitation of the labeled P protein from the cell lysates were carried out by standard procedures (Mazumder *et al.,* 1994). To quantitate the recombinant proteins, a duplicate transfection was performed with each recombinant plasmid in an identical manner except that the [$^{32}$P]orthophosphate

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0042-6822/95 $12.00

was omitted. One-third of the total extract of unlabeled transfected cells from a 33-mm petri dish was concentrated by lyophilization and analyzed by SDS–PAGE followed by Western blot-ECL detection (Amersham). The linear range of detection (approx 5–150 ng) was predetermined by using known amounts of purified bacterial P protein and different times of ECL exposure of the X-ray film as needed. The polyclonal anti-rabbit antibody used in these studies has been described earlier (Mazumder *et al.,* 1994). Where indicated, bands in the autoradiographs (ECL as well as $^{32}$P) were scanned by densitometry (Mazumder *et al.,* 1994).

### Phosphorylation of bacterially expressed P protein *in vitro*

Phosphorylation of bacterially expressed RSV P protein by purified cytosolic CKII has been described previously (Mazumder *et al.,* 1994). In an attempt to simulate *in vivo* phosphorylation, a whole cell extract of uninfected HEp-2 cells was prepared as follows. Freshly grown (75–90% confluent) HEp-2 monolayer in a 10-cm dish was washed twice with ice-cold phosphate-buffered saline (PBS), once with buffer A (50 m*M* Tris–Cl, pH 8.0, 0.1 m*M* EDTA, 10 m*M* NaCl, 5% glycerol), and then scraped into 0.2 ml of buffer A containing 0.5% Tween-20 and 1 m*M* DTT. The suspension was sonicated in ice with intermittent bursts of 5 sec to lyse the cells completely. The resultant lysate, referred to as total or whole cell extract, was used directly as a source of kinase to phosphorylate P protein *in vitro.* Three microliters of extract was used in a 20-$\mu$l standard phosphorylation reaction otherwise identical to the previously published one (Mazumder *et al.,* 1994). Effects of heparin, protamine, and anti-CKII antibody on P protein phosphorylation *in vitro* were studied as described (Mazumder *et al.,* 1994).

Viral L protein was purified from viral nucleocapsids as described (Mazumder and Barik, 1994) and then subjected to a gel filtration chromatography in Sephadex G-100 in the presence of 0.8 *M* NaCl. Reconstituted transcription reactions, standard kinase reactions, and denaturing SDS–PAGE analysis were carried out as described (Mazumder *et al.,* 1994).

### Intracellular turn-over of P protein phosphates

RSV-infected (or mock-infected control) HEp-2 cells (at 12 hr p.i.) were metabolically labeled for 2 hr as described (Mazumder *et al.,* 1994). Following labeling, the radioactive medium was discarded and the monolayer was washed twice with PBS. The radioactivity in P protein was then chased by growing the cells further in nutrient medium containing serum. At different time points of chase, cells were lysed and processed for immunoprecipitation of $^{32}$P-labeled P protein as described (Mazumder *et al.,* 1994). Okadaic acid or cantharidin (at a final concentration of 0.2 $\mu M$ and 5 $\mu M$, respectively), when used, was continuously present from 2 hr prior to the addition of [$^{32}$P]orthophosphate until the harvest of the cells.


FIG. 1. Phosphorylation status of recombinant P proteins. The P protein gene of RSV Long strain and its following mutants were cloned in pcDNA3, expressed in HEp-2 cells and $^{32}$P-labeled as described under Materials and Methods: wild type (lane 2), S237A (lane 3), S232A (lane 4), S232,237A double mutant (lane 5), and S116, 117, 119Ala triple mutant (lane 6). Lane 1 represents immune-precipitates from cells transfected with the vector DNA. A shows the relative amounts of the recombinants determined by Western blot-ECL detection; B presents the $^{32}$P-labeled recombinants analyzed by SDS–PAGE and autoradiography. The numbers below B represent relative amounts of $^{32}$P radioactivity in the bands expressed as percentage of the wild type and normalized against the ECL band intensities of A.

## RESULTS

### Phosphorylation site of recombinant RSV P expressed in eukaryotic cells

RSV P gene and its S232A and S237A mutants were expressed in HEp-2 cells as described under Materials and Methods. The expressed $^{32}$P-labeled P proteins were immunoprecipitated and analyzed by SDS–PAGE and autoradiography. Results presented in Fig. 1 show that the phosphate contents of the S237A and S232A mutants were 72 and 18%, respectively, of the wild-type protein, while that of the S232,237A double mutant was only 6%. Thus, both serines appear to be the sites of phosphorylation, although Ser$^{232}$ contained nearly three times as much phosphate at Ser$^{237}$. A mutant in which three other canonical CKII sites, viz., Ser116, 117, and 119 (Navarro *et al.,* 1991), were mutated to Ala (Mazumder *et al.,* 1994) contained essentially as much phosphate as the wild type (Fig. 1, lane 6), demonstrating that these serines are not detectably phosphorylated *in vivo* as well as *in vitro* (Mazumder *et al.,* 1994; Villanueva *et al.,* 1994).

The demonstration of phosphorylation at Ser$^{232}$ is in apparent variance with our earlier finding that mutation of Ser$^{237}$ to Ala abrogated phosphorylation of bacterially expressed P protein by purified casein kinase II *in vitro.* Therefore, in an attempt to reproduce the *in vivo* phosphorylation *in vitro,* we prepared a total extract of HEp-2 cells, which included not only the cytosolic but the nuclear and particulate (membrane) fractions as well. When used as a potential source of kinase against bacterially made P protein *in vitro,* the whole cell extract indeed phosphorylated the S237A mutant more efficiently than

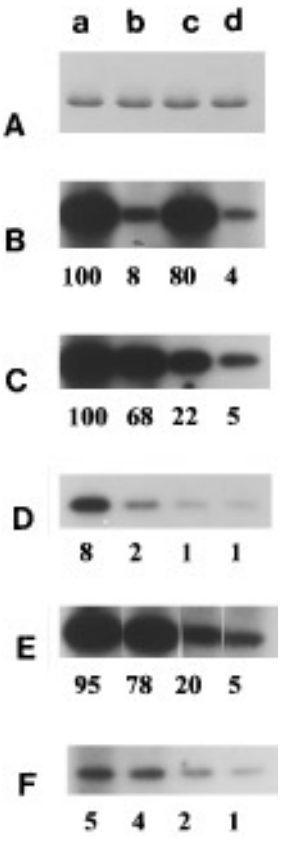


FIG. 2. Phosphorylation of bacterially made RSV P protein *in vitro.* 2 $\mu$g of the following RSV P proteins and its following mutants (Mazumder *et al.,* 1994) were phosphorylated using purified CKII (B) or a total HEp-2 cell extract (C-F) using $\gamma$-$^{32}$P[ATP] as described under Materials and Methods: a, wild type; b, S237A mutant; c, S232A mutant; d, S232,237A double mutant. The phosphorylation reactions were analyzed by SDS–PAGE followed by staining with Coomassie blue and autoradiography. A shows a representative stained protein pattern (this particular one corresponding to C), while C–F shows the autoradiographs. Reactions in D included heparin (1 $\mu$g/ml) and those in E, protamine (1 $\mu$g/ml). In F, the kinase extract was preincubated with 5 $\mu$g anti-CKII antibody for 10 min in ice before use. Numbers at the bottom of panels represent relative radioactivity in each band determined by densitometric scanning; those in B are expressed as percentage of radiolabel in lane a of B while all other numbers are expressed as percentage of label in lane a of C.

the S232A mutant (Fig. 2C). Thus, phosphorylation of bacterially made P protein by a total cell extract *in vitro* approximates the phosphorylation pattern of recombinant P expressed *in vivo.* In order to identify the nature of the kinase, we then examined whether its biochemical properties resemble those of casein kinase II as published (Tuazon and Traugh, 1991; Barik and Banerjee, 1992a; Mazumder *et al.,* 1994). The activity was found to be inhibited by a monospecific anti-CKII antibody (Fig. 2F) and by micromolar concentrations of heparin (Fig. 2D), and either unaffected or slightly stimulated by protamine at a $Mg^{+2}$ concentration of 5 m*M* (Fig. 2E). Since these properties are highly characteristic of CKII (Tuazon and Traugh, 1991), we conclude that the kinase is identical to or very similar to CKII. We would like to emphasize that although our results establish Ser$^{232}$ and Ser$^{237}$ as the major sites of phosphorylation, it is formally possible that small amounts of phosphorylation at other sites may occur under specific conditions.

## Phosphorylation at either Ser$^{232}$ or Ser$^{237}$ activates P protein to different extents

We have recently shown that phosphate-free P protein is transcriptionally inactive *in vitro* and that phosphorylation, primarily at Ser$^{237}$ by purified CKII, can restore activity (Mazumder and Barik, 1994). Thus, the S237A mutant was defective in transcription at all concentrations tested, while the S232A mutant retained substantial activity when 200 ng of it was used in our standard reconstituted transcription reaction *in vitro* (Mazumder and Barik, 1994). Since the Ser$^{232}$ residue appeared to contain the majority (60–70% of wild type) of phosphates *in vivo,* we decided to reexamine the transcriptional activity of the S232A mutant in greater detail. First, we took bacterially made S232A mutant and phosphorylated it at the Ser$^{237}$ residue by using purified CKII (as in Fig. 2B, lane c). In a reciprocal approach, we took the S237A mutant and phosphorylated its Ser$^{232}$ residue using total cell extract (as in Fig. 2C, lane b). The transcriptional activity of the two phosphorylated species was then measured using a range of concentrations of these proteins with constant amounts of L protein and N-RNA template in reconstituted CKII-free transcription reactions (Mazumder and Barik, 1994). Results presented in Fig. 3 show that P protein containing phosphates exclusively at Ser$^{237}$ (denoted by $232^{-}237^{P}$) is transcriptionally defective at low concentrations, whereas at higher concentrations, it displayed transcriptional activity approaching that of the wild type (Mazumder and Barik, 1994). On the other hand, P proteins containing phosphates at Ser$^{232}$ ($232^{P}237^{-}$, $232^{P}237^{+}$ in Fig. 3) exhibited proportional amounts of transcriptional activity at all concentrations tested. The transcriptional defect of the S237A mutant reported earlier was, therefore, most likely due to the lack of its phosphorylation at the Ser$^{232}$ residue, since the kinase activity used in that reaction could not phosphorylate the S237A mutant (Mazumder *et al.,* 1994). Thus, it appears that phosphate groups presented by either Ser$^{232}$ or Ser$^{237}$, regardless of the exact nature of the kinase, can activate the transcriptional property of P protein when used at sufficiently high concentrations. When both serines are devoid of phosphate, either due to a lack of the appropriate kinase activity (Mazumder *et al.,* 1994) or due to mutations in both residues (i.e., S232,237A double mutant in Table 1, line 5), the resultant P protein becomes transcriptionally inactive.

Interestingly, the wild-type bacterial protein, phosphorylated at Ser$^{237}$ (Mazumder *et al.,* 1994) by purified CKII (tentatively denoted as $232^{+}237^{P}$ in Fig. 3), exhibited an

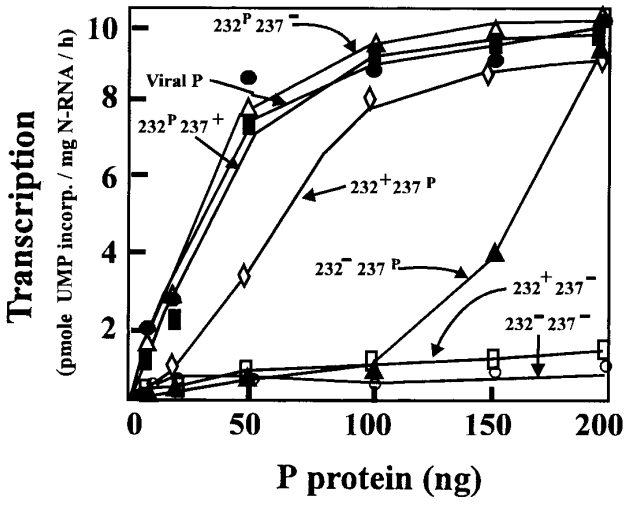


FIG. 3. Transcriptional activity of P proteins of different phosphorylation profiles. Notations used: + and −, wild type (Ser) and mutant (Ala), respectively; P, phosphorylation. *In vitro* transcription reactions were reconstituted as described under Results and Materials and Methods using a range of concentrations (10, 20, 50, 100, 150, and 200 ng) of each P protein as indicated (See Fig. 2 for the phosphorylation status of these proteins): viral protein purified from RSV nucleocapsids (●); wild-type bacterial P, phosphorylated at both $Ser^{232}$ and $Ser^{237}$ by total cell extract ($232^P237^+$, ■); S237A mutant phosphorylated at $Ser^{232}$ by total cell extract ($232^P237^-$, △); wild-type bacterial P phosphorylated by purified CKII ($232^+237^P$; ◇); S232A mutant phosphorylated at $Ser^{237}$ by purified CKII ($232^-237^P$; ▲); phosphate-free S237A mutant ($232^+237^-$; □); S232,237A double mutant ($232^-237^-$; ○). The phosphorylation notations used here are meant primarily for simplicity and are based on the assumption that all phosphorylation in P protein is limited between $Ser^{232}$ and $Ser^{237}$ residues (also see Table 1).

intermediate concentration response of transcription (Fig. 3). In an attempt to determine whether this is possibly due to additional phosphorylation at $Ser^{232}$, we estimated the stoichiometry of phosphates in the various P protein species used in Fig. 3. Results presented in Table 1 show that this species in fact has a significantly higher phosphate content (0.92 mol; line 4) than either single mutant, $232^P237^-$ (0.64 mol; line 2) or $232^-237^P$ (0.71 mol; line 3). The vesicular stomatitis virus (VSV) (NJ) P protein, used as a standard substrate in a parallel reaction, exhibited a stoichiometry of about 1.1 (data not shown). Since the VSV protein is known to be phosphorylated at Ser59 and 61 (Barik and Banerjee, 1992a; Takacs *et al.,* 1992), these results suggest that $232^+237^P$ (Fig. 3) indeed contains some phosphates at $Ser^{232}$, likely at substoichiometric amounts, which may explain its intermediate transcription phenotype.

## Phosphate groups of RSV P protein are stable *in vivo*

We had previously shown that $Ser^{237}$ phosphates are sensitive to calf intestinal phosphatase *in vitro* (Mazumder *et al.,* 1994), which suggested that the phosphates have the potential to turn over *in vivo* and this may provide a mode of regulation of P protein function. However, a direct analysis of the turnover of RSV P protein phosphates has not been performed. To initiate these studies, we first investigated the phosphatase sensitivity of the $Ser^{232}$ phosphates *in vitro.* Bacterially synthesized S237A P protein was $^{32}P$-labeled by total cell extract and used as substrate against calf intestinal phosphatase essentially as described (Mazumder *et al.,* 1994). These studies showed that the $Ser^{232}$ phosphates were as sensitive to phosphatase (data not shown) as were those of $Ser^{237}$. In order to determine whether P protein phosphorylation is stable *in vivo,* we then pulse-labeled RSV-infected cells with [$^{32}$P]orthophosphate and chased the label with time. Results presented in Fig. 4 demonstrate the stability of the phosphates over the time of the experiment (up to 10 hr), suggesting that HEp-2

TABLE 1

Stoichiometry of Phosphorylation of Mutant P Proteins *in vitro*

| Mutant | Kinase source | Possible site(s) of phosphorylation | | mole $PO_4^-$/ mole protein | Transcriptional activity | |
|---|---|---|---|---|---|---|
| | | $Ser^{232}$ | $Ser^{237}$ | | 50 ng P | 200 ng P |
| 1. Wild type | Total CE | +++ | + | 0.85 | 6.3 | 9.8 |
| 2. S237A | Total CE | +++ | − | 0.64 | 7.6 | 10.0 |
| 3. S232A | Purified CKII | − | +++ | 0.71 | 0.7 | 9.2 |
| 4. Wild type | Purified CKII | ++ | ++ | 0.92 | 3.5 | 8.5 |
| 5. S232, 237A | Total CE | − | − | 0.05 | 0.6 | 0.8 |

*Note.* Bacterially expressed P proteins were used as substrate in standard phosphorylation reactions using $\gamma$-$^{32}$P[ATP] of known specific activity as phosphate donor, and either total HEp-2 cell extract (total CE) or purified CKII as kinase (Fig. 3). Plus and minus signs indicate relative amounts of phosphate and are only approximate (See Text). Assignment of phosphates to a particular serine in the wild-type proteins (lines 1, 4) is conjectured from mutational analysis (such as in Fig. 1 and 2). The stoichiometry of phosphorylation was calculated on the basis of radioactivity present in the P protein band and the specific activity of ATP; any contribution of ATP from the cell extract was ignored. The stoichiometry figures were an average of two to three experiments and varied 5–10% between experiments. Transcriptional activity of a given phosphorylated species of P was measured at varying concentrations and plotted in Fig. 3; values for only two concentrations (50 and 200 ng) are shown here in picomoles of [$^{32}$P]UMP incorporated per milligram N-RNA template per hour at 32° (Barik, 1992).

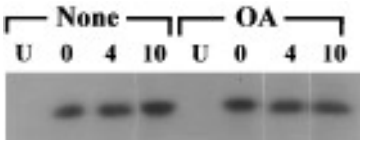


FIG. 4. Phosphate groups of P protein are stable *in vivo.* $^{32}$P-labeled RSV P protein was immunoprecipitated from virus-infected (or mock-infected, lane U) cells at various times of chase (represented by number of hours on each lane: 0, 4, and 10 hr) and analyzed by SDS–PAGE as described under Materials and Methods. An autoradiograph of the gel is shown. OA indicates immunoprecipitates obtained from okadaic acid (0.2 $\mu M$)-treated cells and none from untreated cells.

cells may lack a phosphatase activity that could dephosphorylate RSV P protein. This is further evidenced by the fact that okadaic acid (0.2 $\mu M$) (Fig. 4) and cantharidin (5 $\mu$M, data not shown), established inhibitors of protein Ser/Thr phosphatases, did not appreciably increase the phosphorylation of the P protein (Fig. 4). Thus, the regulation of RSV P protein phosphorylation appears to be different from that of Sendai virus P protein, phosphorylation of which could be stimulated nearly sixfold by treating Sendai-infected cells with phosphatase inhibitors (Byrappa *et al.,* 1995).

### Purified RSV L protein is devoid of P phosphorylating activity

While no RSV-encoded protein kinase has yet been identified, in at least two other negative strand RNA viruses, viz., VSV (New Jersey serotype) and Sendai virus, the viral L proteins appeared to associate with a kinase activity that could phosphorylate the cognate P proteins *in vitro* (Sanchez *et al.,* 1985; Barik and Banerjee, 1992a,b; Einberger *et al.,* 1990; Hammond *et al.,* 1992b). In VSV, protamine inhibited this kinase activity as well as transcription *in vitro* (Barik and Banerjee, 1992a). In view of these findings, we decided to test whether RSV L protein possesses any P protein kinase activity. The final preparation was transcriptionally active when reconstituted with viral P protein, N-RNA template, and cytosolic extract *in vitro* as described previously (Mazumder and Barik, 1994). Kinase activity of this preparation was tested using three kinds of RSV P protein as substrate: bacterially expressed, phosphate-free P protein; bacterially expressed P protein prephosphorylated by total HEp-2 cell extract; and viral P protein purified from mature RS virions. As shown in Fig. 5, L protein failed to phosphorylate any of these P proteins, demonstrating a lack of P kinase activity in L or associated with L. In addition, no phosphorylation could be observed in heparin-treated viral nucleocapsids that contained N-RNA, P as well as L protein (data not shown). Interestingly, protamine at a concentration of 1 $\mu$g/ml still caused about 90% inhibition of RSV transcription reconstituted with this same L preparation *in vitro* (data not shown). Thus, it appears that at least in RSV, the transcription inhibitory effect of protamine is not related to an inhibition of L kinase but perhaps due to the inhibition of the viral polymerase (transcriptase) in a more direct discussion manner.

## DISCUSSION

In this communication, we have undertaken a detailed study of the sites of phosphorylation of P protein *in vivo* as well as the relative roles of the phosphorylated sites in viral transcription. The major conclusions of our studies can be summarized as: (a) Phosphorylation at either Ser$^{232}$ or Ser$^{237}$ activates the transcription activity of P protein (Fig. 3), since complete loss of activity of RSV P protein seems to require lack of phosphate residues at both sites; (b) Phosphate groups at these two sites, however, appear to contribute to the activation process with different efficiency (Fig. 3); (c) Phosphates at Ser$^{232}$ probably play a more important role since they are more abundant *in vivo* (Fig. 1; Sanchez-Seco *et al.,* 1995), are produced more abundantly by crude extracts *in vitro* (Fig. 2), and are transcriptionally more active (Fig. 3) than the Ser$^{237}$ phosphates; (d) Phosphates incorporated in viral P protein in infected cells do not undergo any appreciable turn over (Fig. 4); (e) L protein of RSV is devoid of P protein phosphorylation activity *in vitro* (Fig. 5). The significance of these findings is discussed below.

The most unifying conclusion emerging from the two kinds of concentration kinetics of transcription observed in Fig. 3 is that P proteins containing Ser$^{232}$ phosphates have a substantially higher transcription activity than those with Ser$^{237}$ phosphates, especially discernible at lower concentrations of P. The potent transcription activity of viral P protein (Fig. 3) also suggests that it may be predominantly phosphorylated at Ser$^{232}$. We suspect that either phosphate can impart the proper conformation to

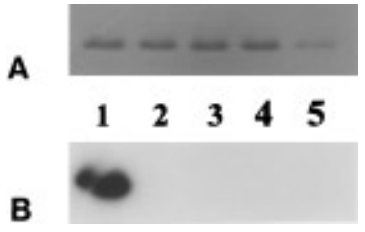


FIG. 5. Lack of P kinase activity in RSV L. 1.5 $\mu$g of bacterially made RSV P protein was used as substrate in standard phosphorylation reactions (Materials and Methods) with the following as potential sources of kinase and $\gamma$-$^{32}$P[ATP] as phosphate donor (except in lane 4, see below)): 1, total cell extract; 2, heparin-treated total cell extract; 3, L protein. In lane 4, P protein was first phosphorylated by total cell extract in the presence of unlabeled ATP; then heparin was added to the reaction, and incubation continued for 2 min; finally, L and $\gamma$-$^{32}$P[ATP] were added and incubation continued for another 15 min. In lane 5, 0.8 $\mu$g viral P protein, purified from RSV nucleocapsids (Mazumder and Barik, 1994) was used as substrate in a standard kinase reaction with L and $\gamma$-$^{32}$P[ATP]. The reactions were analyzed by SDS–PAGE; the stained gel (A) and its autoradiograph (B) are shown. The split band in lane 1 is due to a crack in the gel.

the C-terminal domain of the P protein necessary for transcription, but Ser$^{232}$ phosphates are more effective in this regard. The reasons behind the intermediate transcription activity of P protein phosphorylated by purified CKII and its higher-than-one stoichiometry of phosphates are not clear. The simplest explanation is that purified CKII, following phosphorylation of Ser$^{237}$ *in vitro,* also phosphorylates Ser$^{232}$ to some extent in a cascade manner. While this is under investigation, phosphorylation of Ser$^{232}$ *in vivo* or by total cell extract *in vitro* certainly does not require Ser$^{237}$ phosphates, since the S237A mutant is efficiently phosphorylated (Figs. 1 and 2). An interesting conjecture is that the binding of a cellular protein to the C-terminal domain of P (see below) achieves the same effect as phosphorylation of Ser$^{237}$ and makes the Ser$^{232}$ site accessible for phosphorylation.

At this time, we do not have an explanation for the differential selection of Ser residues by purified cytosolic CKII and the kinase activity in the whole cell extract whose properties as tested are very similar to those of CKII (Figs. 1 and 2). If the cellular kinase is indeed CKII, we speculate the following possibilities to explain their differential activity towards P protein: (a) Nuclear CKII may phosphorylate RSV P protein differently from cytoplasmic CKII. Although the purified CKII used in our studies was obtained from soluble cytosol (Mazumder *et al.,* 1994), recent studies have shown that the bulk of cellular CKII is located in the nucleus, the cytoplasm contributing a much smaller fraction (Krek *et al.,* 1992). It is possible that the two forms of CKII may differ in subtle ways in their recognition of specific substrates. (b) P protein itself may associate with a cellular protein *in vivo,* which alters its recognition by CKII. Clearly, studies of cellular localization of P, a detailed biochemical analysis of the kinase, and the tripartite interaction between the kinase, the P protein, and the cellular protein(s) should help pinpoint the exact mechanism of enzyme-substrate recognition involved in P protein phosphorylation. These analyses are currently under way.

While our studies were in progress, a similar pattern of phosphorylation of RSV P protein was derived by Villanueva and co-workers through a more detailed analysis of recombinant P protein mutants expressed in a vaccinia-based system (Sanchez-Seco *et al.,* 1995). Their studies, as well as ours, pointed to Ser$^{232}$ and Ser$^{237}$ as virtually the only two sites that are phosphorylated by CKII in the recombinant P protein. Thus, in both VSV and RSV, phosphorylation occurs at two neighboring sites, Ser59 and 61 in VSV (Barik and Banerjee, 1992a; Takacs *et al.,* 1992), and Ser232 and 237 in RSV albeit to different extents (Mazumder *et al.,* 1994; Sanchez-Seco *et al.,* 1995; this study). Regardless of the mechanism of site selection, it is noteworthy that both Ser$^{232}$ and Ser$^{237}$ conform to the consensus CKII-site motif (Kennelly and Krebs, 1991) in that they are surrounded by acidic residues with a critical acidic residue (Asp) being at position n + 3, where the Ser is in position n (Fig. 6). In addition, both Ser$^{232}$ and Ser$^{237}$ are highly conserved in P proteins of RSV strains sequenced so far: four human (Satake *et al.,* 1984; Lambden, 1985; Lopez *et al.,* 1988; Johnson and Collins, 1990), one bovine (Mallipeddi and Samal, 1992), and one ovine (Alansari and Potgieter, 1994). As shown in Fig. 6, the neighboring residues of Ser$^{237}$ and those downstream of Ser$^{232}$, including the critical n + 3 residue Asp, are essentially identical in all RSV P proteins, while those just upstream of Ser$^{232}$ are somewhat more variable. It will be interesting to determine the exact sites of CKII-mediated phosphorylation in the other P proteins and compare them with those of the Long strain.

| | |
|---|---|
| **Human** | |
| Long | NDSDNDLSLEDF |
| A2 | NDSDNDLSLDDF |
| Edinburgh | NDSDNDLSLEDF |
| 18537 | NDSDNDLSLDDF |
| **Bovine** | ESSDNDLSLEDF |
| **Ovine** | DNSDNDLSLEDF |

FIG. 6. Sequence conservation around Ser$^{232}$ and Ser$^{237}$ of P protein (see text for references). C-terminal 12 residues in the deduced amino acid sequences of various RSV P proteins are presented; Ser$^{232}$ and Ser$^{237}$ are underlined.

In recent years, P proteins of a number of nonsegmented negative-strand RNA viruses have been shown to serve as substrates for well-characterized cellular kinases *in vitro.* These include: P proteins of VSV (NJ and Indiana serotypes) and RSV (Long), phosphorylated by CKII; and recently, P protein of human parainfluenza virus type 3 (HPIV-3), phosphorylated by the ζ subunit of protein kinase C (De *et al.,* 1995). In VSV (Takacs *et al.,* 1992; Barik and Banerjee, 1992a,b; Gao and Lenard, 1995) and RSV (Mazumder and Barik, 1994), we and others have demonstrated an essential role of this phosphorylation in the transcriptional activity of the P protein *in vitro.* It should be mentioned that in spite of these studies, there is no direct evidence as yet supporting a role of phosphorylation in P activity for any RNA virus *in vivo,* although an indirect evidence is recently provided from studies in HPIV-3 whereby use of a pseudopeptide inhibitor of protein kinase C inhibited viral replication in cell culture (De *et al.,* 1995). Due to the essential nature of CKII in cellular metabolism, selective viral inhibition experiments have not been possible for VSV or RSV. Nevertheless, the stability of the phosphates of the viral P protein in RSV-infected cells (Fig. 4) underscored the potential importance of phosphorylation in viral transcription. Since *in vitro,* P protein can be dephosphorylated by a variety of phosphatases such as calf intestinal phosphatase (Mazumder *et al.,* 1994), bacterial alkaline phosphatase, and lambda phosphatase (Barik, 1993; our unpublished observation), the *in vivo* stability of the phosphates probably owes to the lack of a putative P protein phosphatase. This is further suggested by a lack of increased phos-

phorylation in the presence of phosphatase inhibitors (Fig. 4) and by our inability to dephosphorylate $^{32}$P-labeled P protein *in vitro* using HEp-2 cell extracts (data not shown).

As mentioned earlier, L preparations of VSV (NJ) and Sendai have been reported to contain potent protein kinase activity (L-associated kinase, abbreviated as LAK) that phosphorylates P proteins of these viruses *in vitro.* It has been suggested that this may be an intrinsic property of the L protein rather than a distinct copurified kinase, based primarily on affinity labeling of L proteins of VSV (NJ) and Sendai with ATP analogs (Sanchez *et al.,* 1985; Hammond *et al.,* 1992a), and the biochemical difference between LAK and cellular kinases (Barik and Banerjee, 1992a,b). Furthermore, two lines of indirect evidence suggested a possible role of L-mediated phosphorylation in P function, at least *in vitro:* (i) Mutation of two specific Ser residues to Ala in the P protein of VSV (NJ) abolished its phosphorylation by LAK and its transcription activity (Chattopadhyay and Banerjee, 1987); (ii) LAK could phosphorylate P protein only after phosphorylation of P by CKII; protamine inhibited LAK-mediated phosphorylation of P as well as viral transcription (Barik and Banerjee, 1992a). Our studies reported here show the absence of such a P kinase in RSV L preparations and thus suggest that it may not be important for RSV gene expression. The fact that RSV transcription is still inhibited by protamine raises the possibility that protamine may exert a general inhibition of the RNA-dependent RNA polymerase that is not causally related to an inhibition of L kinase. Although it remains to be determined whether the same may apply for VSV (NJ), studies of VSV (Indiana) transcription complex have either questioned the role of L kinase (Massey *et al.,* 1990; Beckes and Perrault, 1992) or failed to demonstrate a kinase activity associated with highly purified, transcriptionally active L preparations (Massey *et al.,* 1990; Gao and Lenard, 1995), while confirming a role of CKII-mediated phosphorylation of P protein in multimerization and transcription (Barik *et al.,* 1993; Gao and Lenard, 1995). Clearly, the P protein kinase activity associated with L, even if it is an intrinsic property of some L proteins (Einberger *et al.,* 1990; Hammond *et al.,* 1992b), may not be a universal feature of all negative strand RNA viruses.

## ACKNOWLEDGMENTS

We thank Dr. Barsanjit Mazumder for helpful suggestions on eukaryotic expression systems and Dr. Richard E. Honkanen for the kind gifts of okadaic acid and cantharidin. This research was supported by the National Institutes of Health grant AI37938 (to S. B.).

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